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The goal of this research is to develop nuclear medicine imaging agents, for the early detection, diagnosis and treatment of breast cancer, based on the epidermal growth factor receptor (EGFr) biomarker. Our approach is to synthesize small molecule inhibitors of the EGFr tyrosine kinase (tk) suitable for labeling with single photon and positron-emitting radioisotopes and evaluate the imaging potential of these new molecules. We have synthesized and fully characterized ten (10) quinazoline compounds. We have studied their ability to inhibit the EGFr tk in in vitro inhibition assays. All compounds with the exception of one inhibit EGFr tk activity in the nanomolar range. The octanol/water partition coefficients, lipophilicity, of the new compounds have been determined by a high performance liquid chromatography method. The Log P's ranged from 3-5.5. Two compounds have been labeled with iodine-125 and two more have been labeled with fluorine-18. Three routes to the fluorine-18 labeled compounds were explored. We have optimized the synthesis of the iodinated compounds and have initiated binding studies of these compounds in cultured tumor cells. Based on our preliminary results, this class of labeled compounds remains promising as a source of EGFr imaging agents for breast tumors.

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Introduction

The goal of this research is to develop nuclear medicine imaging agents, to aid in the early detection, diagnosis and treatment of breast cancer, based on the epidermal growth factor receptor (EGFr) biomarker. EGF receptors are upregulated in 45% of breast tumors and tumors possessing these receptors fail to respond to conventional treatment correlating with decreased patient survivability. Our approach is to design and synthesize small molecule inhibitors of the EGFr tyrosine kinase (tk) suitable for labeling with single photon and positron-emitting radioisotopes and evaluate the imaging potential of these new molecules. The initial evaluation includes the full chemical characterization of the new molecules, an assessment of their lipophilicity, determination of their ability to inhibit EGFr tk activity and measurement of their affinity for the EGFr tk. Those compounds which possess suitable potency and binding characteristics will be radiolabeled and subjected to a secondary evaluation in in vitro cell studies and in vivo distribution studies in tumor bearing mice. The expected outcome of this research is the development of one or two radiolabeled tracers to be carried forward to preclinical EGFr imaging.

Body

Task 1: Months 1-24: Synthesize and characterize new epidermal growth factor tyrosine kinase inhibitors.

We have synthesized the ten quinazoline analogs shown on the right hand side of Scheme 2. These compounds were designed based on the known structure activity relationships of a series of compounds studied by Parke Davis as potential therapeutic agents for cancer treatment (Bridges, et al., 1996). Another driving consideration in the design of these molecules was the ultimate placement of the radiolabel in such a position that is readily accessible. All of these compounds were synthesized from the common intermediates 4-chloro-6,7-dimethoxy- and 4-chloro-6,7-diethoxy-quinazoline, **4a** and **4b**, respectively.

The synthesis of compounds **4a** and **4b** is shown in Scheme 1. The dimethoxy analog was derived in 2 steps from the commercially available dimethoxyanthranilic acid, **2a**. The diethoxy analog, **4b**, required one extra step starting with the 2-amino-4,5-diethoxy-methyl benzoate which was available from Aldrich Specialty Chemicals. Previously, the route to the diethoxy quinazolines started with the dimethoxyanilino compound. For example, compound **5a** was originally converted into **5b** by removal of the methoxy groups followed by O-ethylation of the hydroxy groups (Bridges, et al., 1996). The overall yield of this two step process was 2%. The synthesis of **4b** represents a new pathway to produce the diethoxy analogs and offers the potential to feasibly produce several new compounds in decent yield.

The synthesis of the ten quinazoline analogs from the common intermediates was quite facile. The appropriate aniline or benzylamine was added to a dimethylformamide solution of **4a** or **4b** and heated for several hours. In many cases the product crystalized out of solution during the reaction. The isolated yields from this series of reactions ranged from 65-90%. All products and intermediates were purified and were chemically characterized by NMR, mass spectroscopy, melting point and elemental analysis. The melting points and elemental analysis results are given in Table 1. All compounds matched their expected carbon, hydrogen and nitrogen (C,H,N) content.

We now have several good candidates for initial biological evaluation and labeling. Thus, over the next 12 months we will produce, as part of this task, a few more quinazoline analogs to complete the series, resynthesize any of those analogs that we need for further evaluation and produce any precursors necessary to carry out the radiolabeling.

Task 2: Months 3-27: Perform in vitro assays to determine tyrosine kinase affinity and inhibition. Measure the lipophilicity of the inhibitors.

We have been fortunate to establish a collaboration with Dr. David Riese at Purdue University. With his help we have determined the EGFr tk inhibition potency of the quinazoline analogs. An additional benefit has been the measurement of the inhibition specificity of the quianazoline analogs for the EGFr tk versus other receptor tyrosine kinases commonly found in breast cancer.

Mouse BaF3 hematopoietic cells that ectopically express either EGFR, ErbB-2 or ErbB-4 were pretreated with the kinase inhibitors. Subsequently, the cells were stimulated with EGF (for cells expressing EGFR) or Neuregulin (for cells expressing ErbB-2 and ErbB-4). The cells were lysed and analyzed for receptor tyrosine phosphorylation by immunoprecipitating with anti-EGFR or anti-ErbB antibodies followed by immunoblotting with an anti-phosphotyrosine antibody.

The results of these assays are given in Table 2. The most potent compound is the 3-bromoanilino-dimethoxy analog **5a**. This corroborates the data found in the literature (Bridges, et al., 1996). Two of the next three most potent compounds are new analogs both suitable for labeling, the iodoanilino-diethoxy analog **7b** and the fluorobenzyl diethoxy analog **9b**. As one can see from the data the inhibition potency of any of the compounds measured against the ErbB2 and ErbB4 tyrosine kinases is one to two orders of magnitude less than the potency for the EGFr tk. Compound **9b** inhibits EGFr tk 50 times better that either ErbB2 tk or ErbB4 tk. This is 3-5 times better than **7b**.

The lipophilicity of the quinazoline analogs was determined by measuring the octanol/water partition coefficients (Log P) for each of the compounds. The HPLC method of Minick, et al. (1993) was used to determine the Log P (Log [octanol/water]) values. The Log P values for all of the quinazoline compounds is given in Table 1. The values range from 3.02 for **9a** to 5.49 for **8b**. The Log P values for compounds **7b** and **9b** are 4.62 and 3.78, respectively. Log P values correlate well with the non-specific binding of the compounds to non-receptor sites. Thus, a compound with a higher Log P might be taken up and remain in non-target tissues increasing the background activity and blurring the image. The best compounds are generally in the range of 2.5-3.5, however we will not know the overall effect of the lipophilicity on the non-specific binding of these new compounds until we look at the biodistribution of the radiolabled compound in small animal models.

We will continue to measure the inhibition, selectivity and lipophilicity of any new analogs developed as part of Task 1. We have initiated studies to develop a radiometric binding assay to assist in the screening of new EGFr tk compounds and we will test the current series of quinazoline analogs in this binding assay. It is possible that the iodo compound, **7b,** labeled with I-125 will be the radioligand for this assay.

Task 3: Months 6-36: Label receptor tracers with fluorine-18, carbon-11, iodine-123, iodine-125, bromine-76. Optimize the synthetic routes for efficient production of high yield, high specific activity tracers.

We have labeled four compounds of this first series of analogs with radioisotopes, two with iodine-125 and two with fluorine-18. A detailed description of the radioiodination of two of the quinaziolines can be found in the manuscript attached as appendix 3. Included is a description of the trimethyltin precursor that was used for the labeling.

The route to the fluorine-18 compounds was a little more interesting. The original route that we had outlined in our proposal (shown as the middle pathway in Scheme 4) did not provide us with the desired compound. The 4-amino-quinazolines, **10a** and **10b** (Scheme 3), were necessary to attempt this first route. The conversion from the chloroquinazolines, **4a** and **4b**, proceeded in good yield. The first radiochemistry step, conversion of the trimethylaminobenzaldehyde to [^{18}F]fluorobenzaldehyde, goes quantitatively, however the reductive coupling to **10a** only yields [^{18}F]fluorobenzylalcohol, not the desired product. The only explanation for this reaction not working is that it does not form the intermediate imine under these conditions. We did attempt a reductive alkyaltion of a simple pyrimidine with [^{18}F]fluorobenzaldehyde and it did not couple either.

The second pathway we attempted was the upper route of Scheme 4. Again the final step was an alkylation of **10a**. The [^{18}F]fluorobenzaldehyde was reduced to [^{18}F]fluorobenzylalcohol and then iodinated with HI to give [^{18}F]fluorobenzyliodide. These two steps were high yielding. Heating the [^{18}F]fluorobenzyliodide with **10a** in triethylamine did not give the final product **[^{18}F]9a**. This reaction may have worked on a longer time scale but fluorine-18 has a two hour half-life and as such long reaction times means less final product.

Having come up short on the other two pathways, we produced the [^{18}F]fluorobenzylamine by the reduction of [^{18}F]fluorobenzonitrile which came from the fluorination of trimethylaminobenzonitrile. The reaction is depicted as the lower route in Scheme 4. The yield of the [^{18}F]fluorobenzylamine was on the order of 50% based on the starting [^{18}F]fluoride ion. The coupling reaction with **4a** and **4b** has been accomplished, albeit at very low yields. This reaction will need some improving in order to produce useful quantities for further study. A detailed abstract on the synthesis of **[^{18}F]9b** can be found in appendix 2.

In the coming year the, we will continue to make improvements on the synthesis of these compounds and develop the methodology to label other promising analogs. Both the iodo and fluoro analogs have displayed the requisite biological characteristics necessary to carry them forward. Also the iodinated analog, **[^{125}I]7b**, might be useful as the radiolabeled tracer in the radiometric binding assay being developed in Task 2.

Task 4: Months 9-36: Evaluate uptake of labeled tracers in tumors possessing differing EGFR titer ranging from 0 to over expression. Determine specific and non-specific binding.

We have initiated studies in this Task, thus these results are very preliminary. We have added **[^{125}I]9a** to whole MB-468 cells (EGFr +) in suspension and aliquots of the media and cells were removed at various times. The cells were separated from the media and each were counted. A significant portion of the activity remained in the media and no change was noted over the time course of the study.

Collaborating with Dr. Buck Rogers at the University of Alabama, we examined the time course of uptake of **[^{125}I]9a** and **[^{125}I]9b** in whole cells that express EGFr (MB468) and those that don't express EGFr (MB453). We also looked at the influence of the presence of EGF in the growth media and the introduction of blocking doses of non-radioactive **5a**. The results were mixed and inconsistent.

A single study was performed using membrane preparations from MB468 cells (EGFr+) and MB453 cells (EGFr -). **[^{125}I]9a** and **[^{125}I]9b** were added to the preparations with and without a blocking dose of **5a**. The results of this study are shown in Table 3. The EGFr positive membranes take up **[^{125}I]9a** and **[^{125}I]9b** and demonstrate receptor mediated uptake in the presence of the blocking compound. The EGFr negative membranes show no binding characteristics. The amount of radiolabel taken up in all cases in the MB453 membranes is equivalent to the background (non-specific) seen in the blocked MB468 membranes.

This membrane preparation study will be the basis for the binding assay to be developed over the next year as part of Task 3. We will continue to examine the uptake of the labeled compounds in the whole cells. It is possible that the iodinated compounds are too lipophilic to cross the cell membranes. Compounds with lesser lipophilicity will be tested.

Key Research Accomplishments

- We have synthesized and fully characterized 10 quinazoline analogs.
- We developed a new synthetic route to produce the diethoxyquinazoline series.
- All analogs, with the exception of one, inhibited epidermal growth factor receptor tyrosine kinase activity at pico- or nano-molar concentrations.
- The iodoanilino- and fluorobenzyl- diethoxyquinazolines demonstrated 10-100 fold selectivity for EGFr tk over ErbB2 tk and ErbB4 tk.
- The lipophilicity of the new analogs ranges from 3-5.5.
- Successfully labeled two analogs with iodine-125 and optimized the reaction yields.
- Successfully labeled two analogs with fluorine-18.

Reportable Outcomes

- Abstract presented at the 13th International Symposium on Radiopharmaceutical Chemistry entitled "Synthesis of 4-(4'-[18F]fluorobenzylamino)-6,7-diethoxyquinazoline: a positron emitting radioprobe for the epidermal growth factor receptor" *J. Lab. Comp. Radiopharm.* **42, Suppl. 1:**S693-S695, 1999. – Appendix 2.
- Manuscript submitted to the Journal of Labeled Compounds and Radiopharmaceuticals entitled "Synthesis of 4-(3'-[125I]iodoanilino-6,7-dialkoxyquinazolines: Radiolabeled epidermal growth factor receptor tyrosine kinase inhibitors" – Appendix 3.

Conclusions

We have made significant progress in many areas of this research. We have designed and synthesized some potent EGFr tk inhibitors that can be readily labeled with imaging radioisotopes. We have begun to evaluate the biological and imaging characteristics of these new inhibitors and have found that they are interacting at the binding site on the tk portion of the receptor. We are on course to continue examining these compounds, as we look towards our first in vivo tests in this coming year. No changes are recommended to the Tasks in the next year.

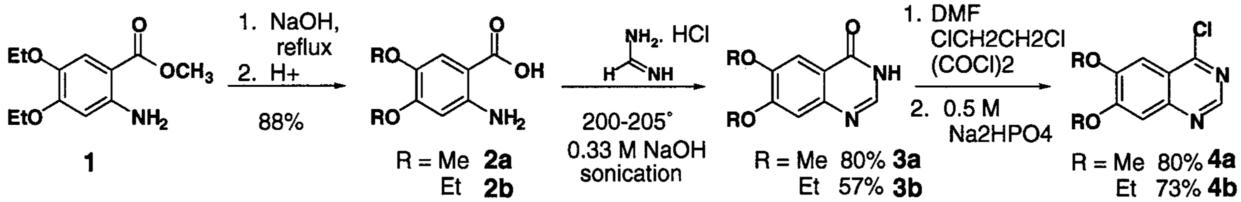
By examining the biological and physical properties of these compounds we may be able to correlate some of their attributes with their therapeutic potential. There are several pharmaceutical companies that are actively pursuing an EGFr based drug for tumor therapy. However, none of the companies are looking at the binding properties of these compounds to the tk receptor, rather they are only interested in their inhibitory value. The inhibition of the signal transduction activity is only one small portion of the problem. If the drug is transported into the cell and only binds to the receptor for a short period of time, it might not be around long enough to repress the cell proliferation effects of the EGF pathway. At this point toxicity and drug delivery become an issue. Although therapeutics is not the focus of this grant the development of a binding assay (Task 2 of this project) that could be used to screen the inhibitors and look at their binding kinetics might be useful in the development of new EGFr-based pharmaceuticals.

References

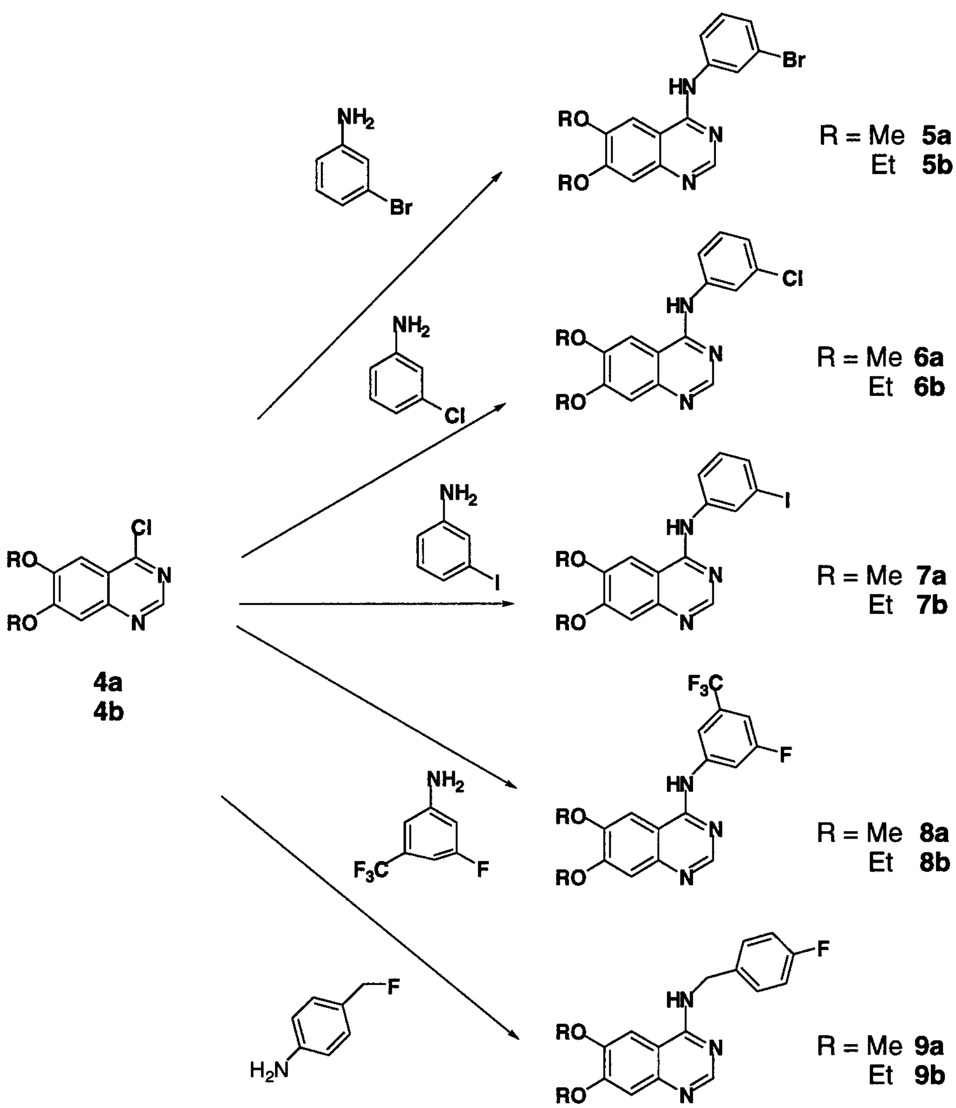
Bridges A.J., Zhou H., Cody D.R., Rewcastle G.W., McMichael A., Showalter H.D., Fry D.W., Kraker A.J., Denny W.A. *J. Med. Chem.* **39:** 267-276 (1996)

Minick, D.J.; Frenz, J.H.; Patrick, M.A.; Brent, D.A. *J. Med. Chem.* **1988**, *31*, 1923-1933.

Appendix 1.

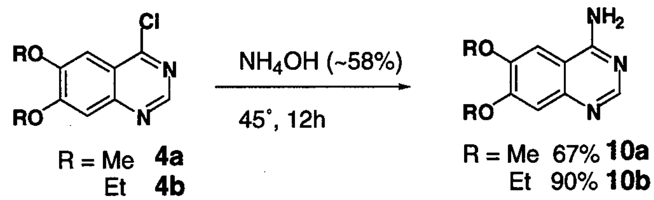


Scheme 1. Synthesis of 4-chloro-6,7-dimethioxy- and 4-chloro-6,7-diethoxy- quinazoline.

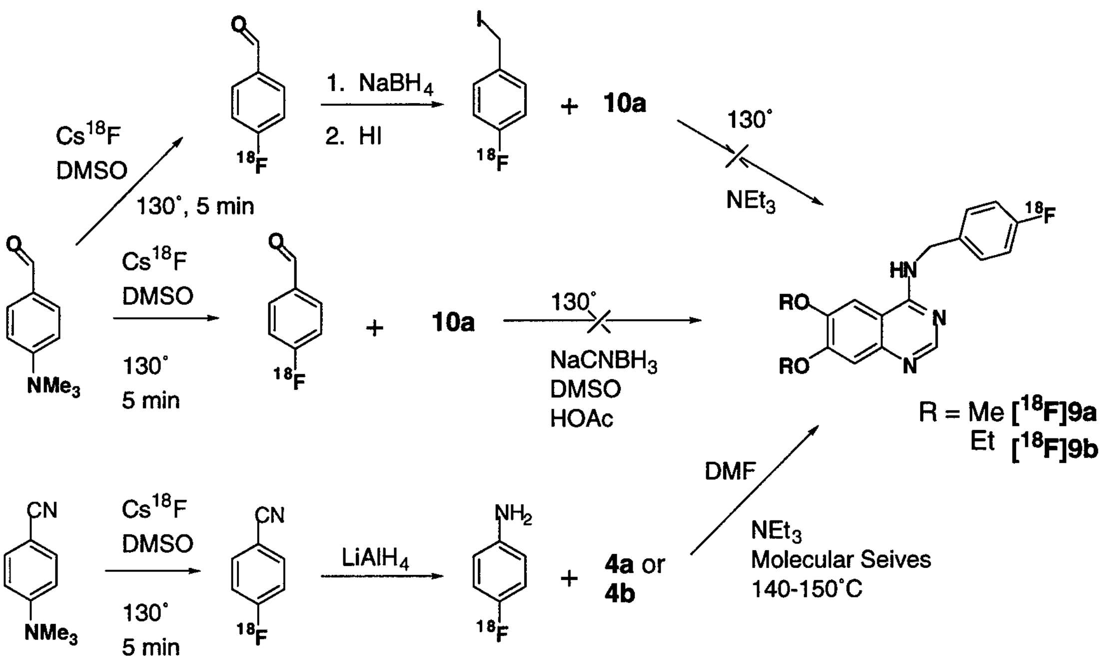


Scheme 2. Synthesis of 4-analinoquinazolines and 4-benzylaminoquinazolines

Appendix 1.



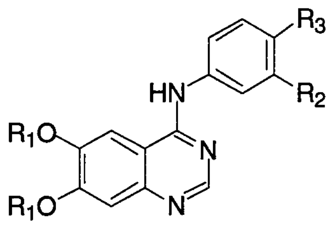
Scheme 3. Synthesis of 4-amino-6,7-dimethoxy- and 4-amino-6,7-diethoxy- quinazoline.



Scheme 4. Synthesis of 4-(4'-[18F]fluorobenzylamino)-6,7-dimethoxy- and 4-(4'-[18F]fluorobenzylamino)-6,7-diethoxy- quinazoline

Appendix 1.

Table 1. Measurement of the lipophilicity (Log P) of the EGFr tk inhibitors.



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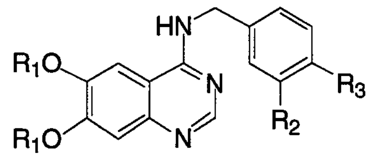

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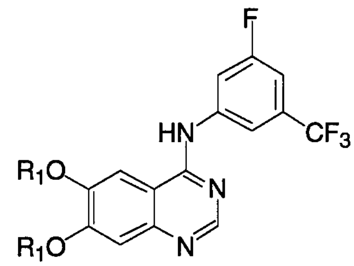

C

no.	type	R_1	R_2	R_3	mp (°C)	formula	analysis	Log P[a]
5a	A	CH_3	Br	H	253	$C_{16}H_{14}BrN_3O_2$	C,H,N	3.49
7a	A	CH_3	I	H	252-253	$C_{16}H_{14}IN_3O_2$	C,H,N	3.65
6a	A	CH_3	Cl	H	249-250	$C_{16}H_{14}ClN_3O_2$	C,H,N	3.51
	A	CH_3	$SnMe_3$	H	225-226	$C_{19}H_{23}N_3O_2Sn$	C,H,N	5.08
5b	A	CH_3CH_2	Br	H	260	$C_{18}H_{18}BrN_3O_2$	C,H,N	4.40
7b	A	CH_3CH_2	I	H	265-266	$C_{18}H_{18}IN_3O_2$	C,H,N	4.62
6b	A	CH_3CH_2	Cl	H	260-261	$C_{18}H_{18}ClN_3O_2$	C,H,N	4.31
	A	CH_3CH_2	$SnMe_3$	H	154-156	$C_{21}H_{27}N_3O_2Sn$	C,H,N	5.85
9a	B	CH_3	H	F	256-257	$C_{17}H_{16}FN_3O_2$	C,H,N	3.02
9b	B	CH_3CH_2	H	F	243	$C_{19}H_{20}FN_3O_2$	C,H,N	3.78
8a	C	CH_3			265-267	$C_{19}H_{17}F_4N_3O_2$	C,H,N	4.66
8b	C	CH_3CH_2			272	$C_{17}H_{13}F_4N_3O_2$	C,H,N	5.49

[a] The Standard Deviation for the Log P values measured by the HPLC method is ± 6% based on propagation of errors.

Appendix 1.

Table 2. Inhibition of tyrosine kinase phosphorylation (IC50 values)

Compound No.	EGFr tk [nM]	ErbB2 tk [nM]	ErbB4 tk [nM]
5a	1.8 ± 1.4	143 ± 52	49 ± 16
5b	4.4 ± 3.2	192 ± 90	57 ± 25
7a	11 ± 6.4		
7b	4.6 ± 3.9	69 ± 10	90 ± 41
8a	19 ± 6.5		
8b	>100		
9a	13 ± 5.8		
9b	6.6 ± 4.2	282 ± 152	>300

Table 3. Receptor mediated binding of the EGFr tk inhibitors

	Percent of Radiolabel bound to membranes			
Radiolabel	MB468 (EGFr+) Membranes		MB453 (EGFr-) Membranes	
	(-)**5a**^{x}	(+)**5a**^{y}	(-)**5a**^{x}	(+)**5a**^{y}
[^{125}I]9a	72%	26%	28%	29%
[^{125}I]9b	65%	33%	32%	37%

^{x} No blocking dose added to the membranes.

^{y} Receptor blocking dose added to the membranes.

Appendix 2.

SYNTHESIS OF 4-(4′-[^{18}F]FLUOROBENZYLAMINO)-6-7-DIETHOXYQUINAZXOLINE: A POSITRON EMITTING RADIOPROBE FOR THE EPIDERMAL GROWTH FACTOR RECEPTOR

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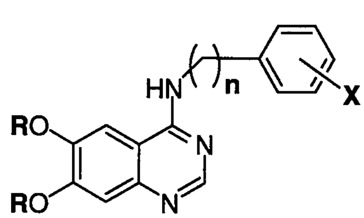
Key Words: epidermal growth factor receptor, tyrosine kinase, inhibitor, quinazoline, fluorine-18, PD15305

The oncogenic epidermal growth factor receptor (EGFr) is a transmembrane protein with an extracellular binding domain and an intracellular tyrosine kinase region. Binding of EGF to the receptor initiates the phosphorylation of tyrosine by the tyrosine kinase, propagating a signal that ultimately stimulates cell growth and proliferation.(1) EGFrs are upregulated in several different tumors including lung cancer, endometrial cancer and breast cancer.(2) Our interest lies in developing EGFr-based imaging agents for breast cancer, where EGFr is upregulated in 45% of the tumors and correlated with poor prognosis.(3,4)

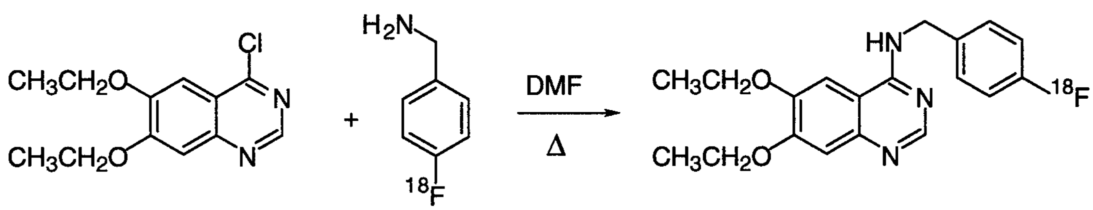
Inhibitors of the receptor tyrosine kinase are target drug candidates for anticancer therapy. A class of EGFr tyrosine kinase inhibitors, 4-anilinoquinazolines, has been shown to be particularly potent and selective.(5) One analog, 4-(3′-bromoanilino)-6,7-dimethoxyquinazoline (**1**, PD153035), inhibits EGFr activity with an IC_{50} value of 25 pM (free receptor assay) and has progressed to phase I clinical trials.(6) The radioiodinated 3'-iodo analog of **1** has shown receptor mediated uptake in the human tumor cell line MDA-468.(7) Two positron emitting labeled quinazoline analogs, [^{18}F]fluoroethoxy (8) and [^{11}C]methoxy (8-10), have been reported. Preliminary biodistribution studies have shown differential uptake of the carbon-11 compound in implanted tumors versus healthy tissue, demonstrating potential as a tumor imaging agent.(9)



COMPD	R	n	X	IC_{50} (nM)
1	Me	0	3'-Br	2.38 ± 1.27
2	Me	1	4'-F	14.02 ± 5.18
3	Et	0	3'-Br	5.91 ± 3.41
4	Et	1	4'-F	7.57 ± 4.64

We have synthesized a series of fluorine and iodine quinazoline compounds suitable for radiolabeling and assessed their ability to inhibit EGFr tyrosine kinase activity.(11) Two of the fluorine compounds, **2** and **4**, are shown above along with their respective IC_{50} values from an *in vitro* assay using whole cells. Compounds **1** and **3** are shown for comparison. There is a loss of inhibition in the transition from 3′-bromoanilino to the 4′-fluorobenzyl while an increase in inhibition from the methoxy to ethoxy is seen within the fluorine series. Compound **4** also demonstrates better inhibition than the 3′-iodo compounds (data not shown).

We previously reported the preparation of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-dimethoxyquinazoline, **[^{18}F]2**, by coupling [^{18}F]fluorobenzylamine with 6,7-dimethoxychloroquinazoline.(11) [^{18}F]fluorobenzylamine was prepared by reduction of the corresponding benzonitrile precursor with lithium aluminum hydride. This method necessitated a substantial synthetic work-up to purify the intermediate [^{18}F]fluorobenzylamine. We have since adapted a recent literature procedure (12) whereby the nitrile moiety of *p*-[^{18}F]fluorobenzonitrile was reduced with borane-dimethylsulfide resulting in high radiochemical purities (>95%, determined by thin-layer radiochromatography) in a much shorter time and with a simplified workup. Our only deviation from the reported reaction was the use of Cs[^{18}F] as the nucleophilic fluoride ion in the reaction with 4-*N,N,N*-trimethylammonium-benzonitrile triflate (0.5 mg). The [^{18}F]fluorobenzylamine was purified on a C_{18} Sep Pak® cartridge and eluted with DMF. The first 0.5 mL of DMF from the Sep Pak® was discarded.



5 **6** **[^{18}F]4**

The preparation of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-diethoxyquinazoline **[^{18}F]4** is shown in the scheme above. The [^{18}F]fluorobenzylamine **6**/ DMF solution was added to 4-chloro-6,7-diethoxyquinazoline **5** (0.5 mg) and proton sponge (0.5 mg) followed by heating at 140 °C for 45-60 minutes. The solution was applied to a C_{18} Sep Pak® cartridge and eluted with methanol. The methanolic solution was diluted with water and purified by reversed phase HPLC (Whatman M9/50 ODS3 semi-preparative column 65:35 methanol/water, pH 7.40, 6 mL/min). The desired **[^{18}F]4** eluted at 14 minutes. The total synthesis time was approximately 2.5 hours from the end of bombardment. While the radiochemical purity was >99%, the overall yield was low.

We have successfully synthesized 4-(4'-[^{18}F]-fluorobenzylamino)-6,7-diethoxyquinazoline and are continuing to improve the radioligand synthesis for further evaluation of this EGFr ligand as a potential tumor imaging agent.

Acknowledgments

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Appendix 3.

SYNTHESIS OF 4-(3'-[^{125}I]IODOPHENYLANILINO)-6,7-DIALKOXYQUINAZOLINES: RADIOLABELED EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS.

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SUMMARY

The preparation of two radioiodinated analogs of the epidermal growth factor-tyrosine kinase (EGFrTK) inhibitor *PD153035* (4-(3'-bromoanilino)-6,7-dimethoxyquinazoline) are reported herein. The two analogs, 4-(3'-[^{125}I]iodoanilino)-6,7-dimethoxyquinazoline and 4-(3'-[^{125}I]iodoanilino)-6,7-dimethoxyquinazoline were synthesized *via* iododestannylation of the corresponding 4-(3'-trimethylstannylanilino)-6,7-dialkoxyquinazolines to form the desired I-125 labeled products in good yield and high radiochemical purity (>99%).

KEY WORDS: epidermal growth factor receptor, iodine-125, PD153035, quinazoline, tyrosine kinase inhibitor.

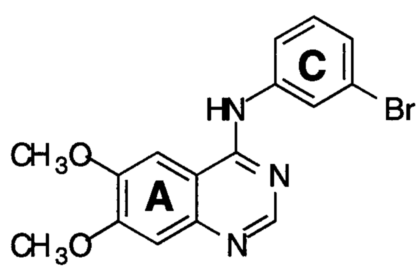
INTRODUCTION

Protein tyrosine kinases (PTKs) regulate cell division, growth and differentiation. Activation of the PTKs is one of the first steps in the signal cascade that initiates these cellular processes. The epidermal growth factor receptor (EGFr) is a member of a family of PTK-linked receptors where the tyrosine kinase domain is an integral part of the receptor. The EGFr is a 170 kD transmembrane protein possessing an extracellular ligand binding domain and a intracellular tyrosine kinase domain. The binding of EGF to the ligand domain of two adjacent receptors promotes a conformational change that brings the two receptors together. The dimerization

activates the tyrosine kinase towards phosphorylation of tyrosine residues on the adjacent receptor (interphosphorylation) as well as phosphorylation of other enzymes, thus propagating the signal throughout the cell (1-3).

The aberrant expression and activation of growth factor receptors in normal cells has been implicated in the promotion and proliferation of malignant growths (4). EGFr overexpression has been noted in a number of human neoplastic lesions (5) including lung cancer, endometrial carcinoma and breast cancer (6). Therapeutic response and patient survival has been negatively correlated with EGFr upregulation (7, 8). Small molecules capable of selective inhibition of the EGFr activation have been the target of intense research over the last several years in an effort to develop a therapeutic antitumor drug (9-11). Based on the concentration of receptors in the tumors, growth factor receptors have been sited as potential targets for imaging as well as radiotherapeutic agents (12).

Several classes of compounds are being investigated as tyrosine kinase inhibitors (11). One class, 4-anilinoquinazolines, has been shown to be particularly potent and selective ATP site inhibitors (13). The most potent member of this class, 4-(3'-bromoanilino)-6,7-dimethoxyquinazoline (PD153035, **1**), inhibits EGFr phosphorylation with a K_i of 5 pM and has demonstrated selectivity with mM inhibition of other growth factor receptors(14).



1, PD153035

Figure 1. 4-(3'-bromoanilino)-6,7-dimethoxyquinazoline, PD153035

Several investigators have initiated programs to develop imaging agents based on the small molecule EGFr inhibitors. A number of radiolabeled analogs of PD153035 have been reported in prefatory communications. The compounds incorporate labeled substituents on the A or C rings of the quinazoline (see Figure 1). The C ring substituted analogs include 4-(3'-[^{125}I]iodoanilino)- (15), 4-(3'-[^{18}F]fluoro-5'-trifluoromethylanilino)- (16), 4-(3',4'-dichloro-6'-[^{18}F]fluoroanilino)- (16), and 4-(4'-[^{18}F]fluorobenzyl)-dimethoxyquinazolines (17) and 4-(4'-[^{18}F]fluorobenzyl)-diethoxyquinazoline (18). The 7-[^{18}F]fluoroethoxy- (19) and the 6-or 7-

[^{11}C]methoxy- (19, 20) constitute the A ring labeled analogs. Preliminary in vitro studies with the 3'-[^{125}I]iodo analog demonstrated receptor mediated uptake in cells containing high EGFr titer (15). A more recent study of the ^{11}C-methoxy derivative demonstrated some uptake in human neuroblastoma xenographs in mice (21, 22). While neither of these studies were unequivocal, the evidence suggests that further studies towards the development of in vivo imaging agents for EGFr expression in tumors is warranted

As part of ongoing effort to develop positron-emitting EGFr imaging agents in our laboratory, we sought to produce a labeled compound for use in radiometric binding studies. To this end we report herein the detailed synthesis of two iodine-125 labeled analogs of PD153035.

RESULTS and DISCUSSION

The two iodinated analogs presented were chosen for labeling based on known structure activity relationships derived from the inhibition of EGFr tyrosine kinase activity by numerous analogs of PD153035 produced by Parke Davis et al. (23). The investigators found that replacing the bromine on PD153035 with iodine increased the IC_{50} value 35 times, albeit the iodo compound still retained subnanomolar inhibition of the EGFr tyrosine kinase (IC_{50} = 0.89 nM). Replacement of the 6 and 7 methoxy moieties with 6 and 7 ethoxy groups decreased the IC_{50} of PD153035 nearly 5 fold, 0.025 nM to 0.006 nM. Based on these data we prepared the 3'-iodophenyl-6,7-diethoxy analog and labeled it with ^{125}I as well as the corresponding 6,7-dimethoxy derivative.

The synthetic route for the preparation of the analinoquinazolines is outlined in Figure 2. The common intermediates 4-chloro-6,7-dimethoxy- , **2a**, and 4-chloro-6,7-diethoxyquinazoline, **2b**, were used to prepare the nonradioactive iodine compounds and the trialkylstannyl precursors for labeling. The procedure outlined by Bridges, et al. (23) was followed for the synthesis of **2a** starting from the commercially available dimethoxyanthranilic acid. The corresponding diethoxyanthranilic acid was not commercially available, however it was produced in one step by the saponification of the available 2-amino-4,5-diethoxy-methyl benzoate. The synthesis of **2b** proceeded analogously to that of **2a** (24).

The bromo- and iodo-anilino analogs, **1**, **3**, **5a** and **5b**, were produced by heating the appropriate bromo- of iodo-aniline with the respective chloroquinazoline, **2a** or **2b**, in anhydrous DMF. This reaction proceeded very cleanly and gave high yields

(84-91%) of the haloanilino compounds. This compares favorably to the reactions reported by Bridges, et al. (23) where they used isopropanol as the solvent. The bromoanilino compounds, **1** and **3**, were converted into the corresponding trimethylstannyl derivatives by reacting with hexamethylditin in the presence of a catalytic amount of tetrakistriphenylphosphinepalladium(0) in 55-60% yield.

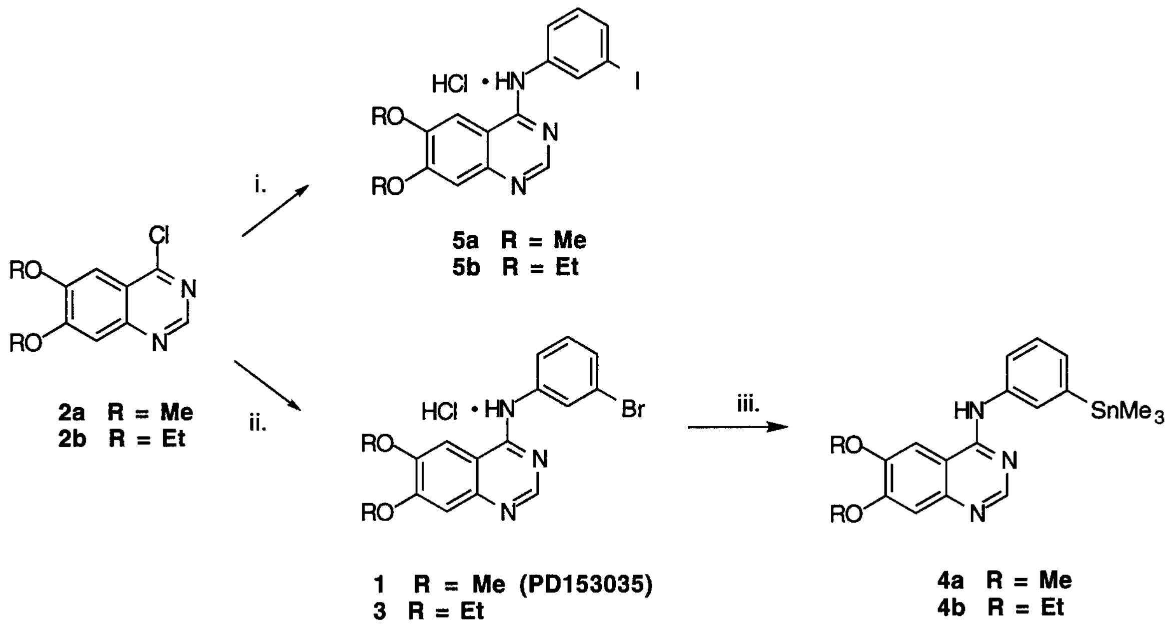


Figure 2. Synthetic route for the iodoanilino- and trimethylstannylanilino-dialkoxyquinazolines. (i.) 3-iodoaniline, DMF, Δ; (ii.) 3-bromoaniline, DMF, Δ; (iii.) $(SnMe_3)_2$, $Pd(Ph_3)_4$, dioxane.

The conversion of the stannyl compounds to the desired ^{125}I labeled quinazolines is shown in Figure 3. The iododestannylation reaction proceeded rapidly, less than 15 minutes, using $Na^{125}I$ in the presence of dichloramine-T. Purification by solid phase extraction (reversed-phase, C18) and HPLC gave either **^{125}I-5a** or **^{125}I-5b** in 40-55% radiochemical yield. The radiochemical purity of **^{125}I-5a** and **^{125}I-5b** was greater than 98% by TLC.

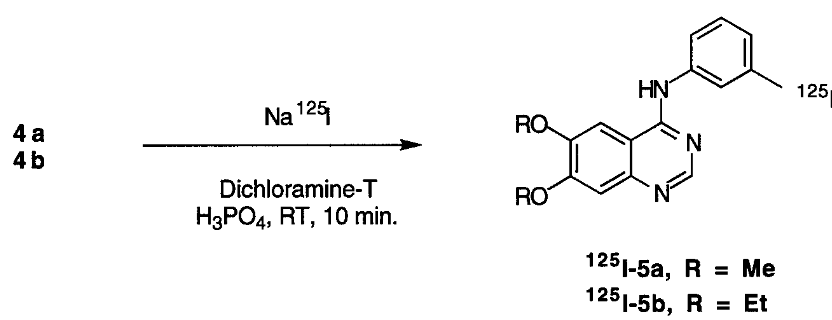


Figure 3. Radiosynthesis of 3'-[^{125}I]iodo-quinazoline analogs of **5a** and **5b**.

EXPERIMENTAL

All chemicals were purchased from Aldrich Chemical Co. and used without further purification. The 2-amino-4,5-diethoxy-methyl benzoate was purchased from Aldrich Specialty Chemicals. NMR spectra were obtained on a Bruker VBAMX400 400 MHz spectrometer. Elemental analyses were performed by the Microanalytical Laboratory at the College of Chemistry, University of California, Berkeley. Melting points were taken on a Mel-Temp apparatus and are reported uncorrected. Mass Spectra were obtained on a Perkin Elmer SCIEX spectrometer at the SynPep Corporation.

4-(3'-bromoanilino)-6,7-dimethoxyquinazoline (1).

The preparation of **1** followed the method described below for **3**. Yield (87%). ^{1}H NMR spectra were identical to those reported in the literature (23).

4-(3'-bromoanilino)-6,7-diethoxyquinazoline (3).

A clear, pale yellow anhydrous DMF solution (3 mL) of 4-chloro-6,7-diethoxyquinazoline **2b** (0.1 g, 0.396 mmol) was combined with 3-bromoaniline (64.6 μL, 1.5 equiv.) to form a clear pale pink solution. Within 15 minutes of heating the reaction flask at 80° C under argon, precipitation of a white solid was observed. The heterogenous solution was heated for an additional 45 minutes, then cooled to room temperature for 15 minutes. The solid was filtered and washed with ethyl acetate (20 mL) to give the hydrochloride salt of **3b** as a bright white, pulpy solid. Yield 0.15 g (89%). m.p. 260° C. ^{1}H NMR ($CDCl_3$): δ 8.63 (*s*, 1H, Ar*H*), 7.88 (*s*, 1H, Ar*H*), 7.87 (*s*, 1H, Ar*H*), 7.59, (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.38 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.31 (*t*, 1H, ArH, J = 8.0 Hz), 4.18 (*m*, 4H, OCH_2CH_3), 1.42 (*m*, 3H, OCH_2CH_3). APCI Mass Spec 388.1, 390.1 [M+1]. Elemental Analyses $C_{18}H_{19}BrClN_3O_2$ calcd. C 50.90, H 4.51, N 9.89; found C 49.32, H 4.66, N 9.44.

4-(3'-trimethylstannylanilino)-6,7-dimethoxyquinazoline (4a).

The preparation of **4a** starting from 40 mg (0.11 mmol) of the free base of **1** was carried out in a manner analogous to **4b.** Yield 30 mg (61%) ^{1}H NMR ($CDCl_3$): δ 8.64 (*s*, 1H, Ar*H*), 7.73 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.62 (*s*, 1H, Ar*H*), 7.40 (*t*, 1H, Ar*H*, J = 8.0 Hz), 7.28 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.26 (*s*, 1H, Ar*H*), 7.02 (*s*, 1H, Ar*H*), 4.02 (*s*, 3H, OCH_3). APCI Mass Spec 446.1, 444.0, 442.1 [M+1]. Elemental Analyses $C_{19}H_{23}N_3O_2Sn$ calcd. C 51.38, H 5.22, N 9.46; found C 51.59, H 5.47 N 9.12.

4-(3'-trimethyltstannylanilino)-6,7-diethoxyquinazoline (4b).

The hydrochloride salt of **2b** was first converted to the free base by partitioning the solid between ethyl acetate (3 mL) and 1*N* sodium hydroxide (2 mL). After thoroughly shaking the two layers, the ethyl acetate layer was separated and the aqeous base solution was extracted with addition ethyl acetate (2 x 3 mL). The ethyl acetate fractions were pooled, dried over magnesium sulfate, filtered, and solvent removed *in vacuo* to give an oil, then recrystallized from diethyl ether to give white crystals (yield 60 mg, 66%). A toluene solution (5 mL) containing the free base **2b** (75 mg, 0.493 mmol), hexamethylditin (48 μL, 1.2 equiv.) and a catalytic amount of tetrakistriphenyl-phosphine palladium(0) (22.3 mg, 10 mol%) was heated at 105° C under Argon for 16 hours resulting in an intense, dark black solution. The palladium catalyst was removed by eluting through a short silica pad to give a clear yellow solution. The solution was concentrated *in vacuo*, then purified by radial chromatography (2 mm silica plate thickness; eluted first with hexanes, then gradually adjusted to 30% ethyl acetate/hexane, and finally 100 % ethyl acetate). Purity was confirmed by a single spot in TLC (silica, 75% ethyl acetate/hexane, R_f 0.55). Yield 50 mg (56%) ^{1}H NMR ($CDCl_3$): δ 8.62 (*s*, 1H, Ar*H*), 7.75 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.61 (*s*, 1H, Ar*H*), 7.39 (*t*, 1H, Ar*H*, J = 8.0 Hz), 7.27 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.23 (*s*, 1H, Ar*H*), 7.02 (*s*, 1H, Ar*H*), 4.22 (*m*, 4H, OCH_2CH_3), 1.55 (*m*, 3H, OCH_2CH_3). APCI Mass Spec 474, 472.1, 470 [M+1]. Elemental Analyses $C_{21}H_{27}N_3O_2Sn$ calcd. C 53.42, H 5.76, N 8.90; found C 53.76, H 6.06 N 8.54.

4-(3'-Iodoanilino)-6,7-dimethoxyquinazoline (5a).

The preparation of **5a** followed the method described above for **3**. The chloroquinazoline **2a** (0.28 g, 1.22 mmol) was reacted with 1.5 equiv. of iodoaniline to yield 0.5 g (92%) of **5a**. m.p. 252-253° C. ^{1}H NMR (DMSO): δ 7.91 (*s*, 1H, Ar*H*), 7.31 (*s*, 1H, Ar*H*), 7.16 (*s*, 1H, Ar*H*), 6.80 (*d*, 1H, Ar*H*, J = 8.0 Hz), 6.70 (*d*, 1H, Ar*H*, J = 8.0 Hz), 6.36 (*s*, 1H, Ar*H*), 6.32 (*t*, 1H, Ar*H*, J = 8.0 Hz), 3.05 (*s*, 3H, OCH_3), 3.03 (*s*, 3H, OCH_3). APCI Mass Spec 408.0 [M+1]. Elemental Analyses $C_{16}H_{15}ClIN_3O_2$ calcd. C 43.31, H 3.41, N 9.47; found C 43.66, H 3.45, N 9.51

4-(3'-Iodoanilino)-6,7-diethoxyquinazoline (5b)

The preparation of **5b** followed the method described above for **5a**. The chloroquinazoline **2b** (0.12 g, 0.46 mmol) was reacted with 1.5 equiv. of iodoaniline to yield 0.19 g (88%) of **5b**. m.p. 265-266° C. ^{1}H NMR (DMSO): δ 8.86 (*s*, 1H, Ar*H*), 8.20 (*s*, 1H, Ar*H*), 8.10 (*s*, 1H, Ar*H*), 7.75 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.67 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.30 (*s*, 1H, Ar*H*), 4.27 (*m*, 4H, OCH_2CH_3), 1.44 (*m*, 3H,

OCH_2CH_3). APCI Mass Spec 436.0 [M+1]. Elemental Analyses $C_{18}H_{19}ClIN_3O_2$ calcd. C 45.83, H 4.06, N 8.91; found C 46.11, H 3.45, N 8.85

4-(3'-[^{125}I]Iodoanilino)-6,7-dimethoxyquinazoline ([^{125}I]-5a).

The preparation of ***[^{125}I]-5a*** followed the method outlined in ***[^{125}I]-5b***. Beginning with 0.95 mCi of $Na^{125}I$, a 0.5 mCi (53%) yield of ***[^{125}I]-5a*** was obtained after HPLC purification (C_{18} analytical column, 50% MeOH/water final pH 7.40, flow= 2mL/min., Retention volume = 50-69 mL, Retention time 25-34.5 min).

4-(3'-[^{125}I]Iodoanilinio)-6,7-diethoxyquinazoline ([^{125}I]-5b).

A 1-dram screw-cap vial equipped with a teflon cap and stir bar containing acetonitrile (200 μL) was charged with 3'-trimethylstannane-6,7-dimethoxyquinazoline (~ 0.5 mg). To the vial was added a solution of $Na^{125}I$ (~1 mCi) followed by the addition of 85% phosphoric acid (50 μL) and dichloramine-T (20 μL). After reacting at room temperature for 10-15 minutes, the reaction was quenched with 10% $Na_2S_2O_5$ (50 μL). The quenched vial solution diluted with 25 mL of 17 MΩ H_2O then eluted through an activated C_{18} Sep Pak to trap >95% of the measured activity. The cartridge was then eluted with 0.2 cc of methanol (0.1 μCi) then an additional 1.6 cc of methanol (936 μCi). The second elution fraction of methanol was slowly evaporated under a gentle stream of Argon. The evaporated residue was taken back up in 2 mL of a buffered methanol/water solution (from phosphoric acid/triethylamine) and chromatographed by HPLC (C_{18} analytical column, 50% MeOH/water final pH 7.40, flow= 2mL/min., Retention volume = 106-140 mL, Retention time 53-70 min). The product was concentrated by trapping on an activated C18 Sep Pak. ***[^{125}I]-5b*** (1.3 mCi, 45% yield) was collected in 1 mL of methanol eluent.

CONCLUSION

We have successfully labeled two EGFr tyrosine kinase inhibitors with iodine-125. The compounds were produced in sufficiently high yield and radiochemical purity. Both these compounds demonstrated receptor mediated affinity for the EGFr tyrosine kinase binding site in initial binding studies. One of these compounds may find utility in the development of a radiometric binding assay for the measurement of the receptor binding affinity of novel EGFr inhibitors.

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